

## REVIEW ARTICLE

# Cre Recombinase: The Universal Reagent for Genome Tailoring

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## INTRODUCTION

In September 1998 the workshop for “Conditional Genome Alterations” in Cold Spring Harbor had a simple but important take-home message: “Cre works” (Rossant and McMahon, 1999). After several years of careful investigations, geneticists are now certain that the Cre recombinase of the P1 bacteriophage efficiently catalyzes recombination between two of its consensus 34 base pair DNA recognition sites (*loxP* sites) in any cellular environment and on any kind of DNA. Obviously mouse geneticists, including myself, are among those who are most excited about this finding. The reason is obvious: When we acquired the Cre/*loxP* recombinase system as a tool, we reached the level of sophistication of “no more practical limitation” in tailoring the mouse genome.

The well-established practice of transgenesis allows the addition of functional DNA pieces into the mouse genome. Furthermore, with the advent of mouse embryonic stem (ES) cells and homologous recombination-based gene targeting, we are capable of removing any existing gene from the genome. By adding the Cre recombinase system, we can excise or invert *loxP*-flanked DNA segments or create intermolecular recombination between different DNA molecules. By combining all of these tools, we are capable of creating any desired modification of the mouse genome, from introducing specific point mutations to large site-specific chromosomal aberrations. Furthermore, using the recombinase activity as a genetic activation or inactivation switch, conditional transgenesis or conditional knock-outs became available. The strategies are designed; the proof of principles are given. Many of these strategies utilize a common set of reagents, namely transgenic mouse lines that express the Cre recombinase in a tissue-specific manner and with high fidelity with respect to the recombinase activity. The bottleneck for taking full advantage of these powerful technologies seems to be the realization of this universal set of reagents, the Cre transgenic lines.

The purpose of this introductory review in this special issue of *Genesis* is to summarize the recent status of mouse genetic strategies that apply the Cre recombinase system.

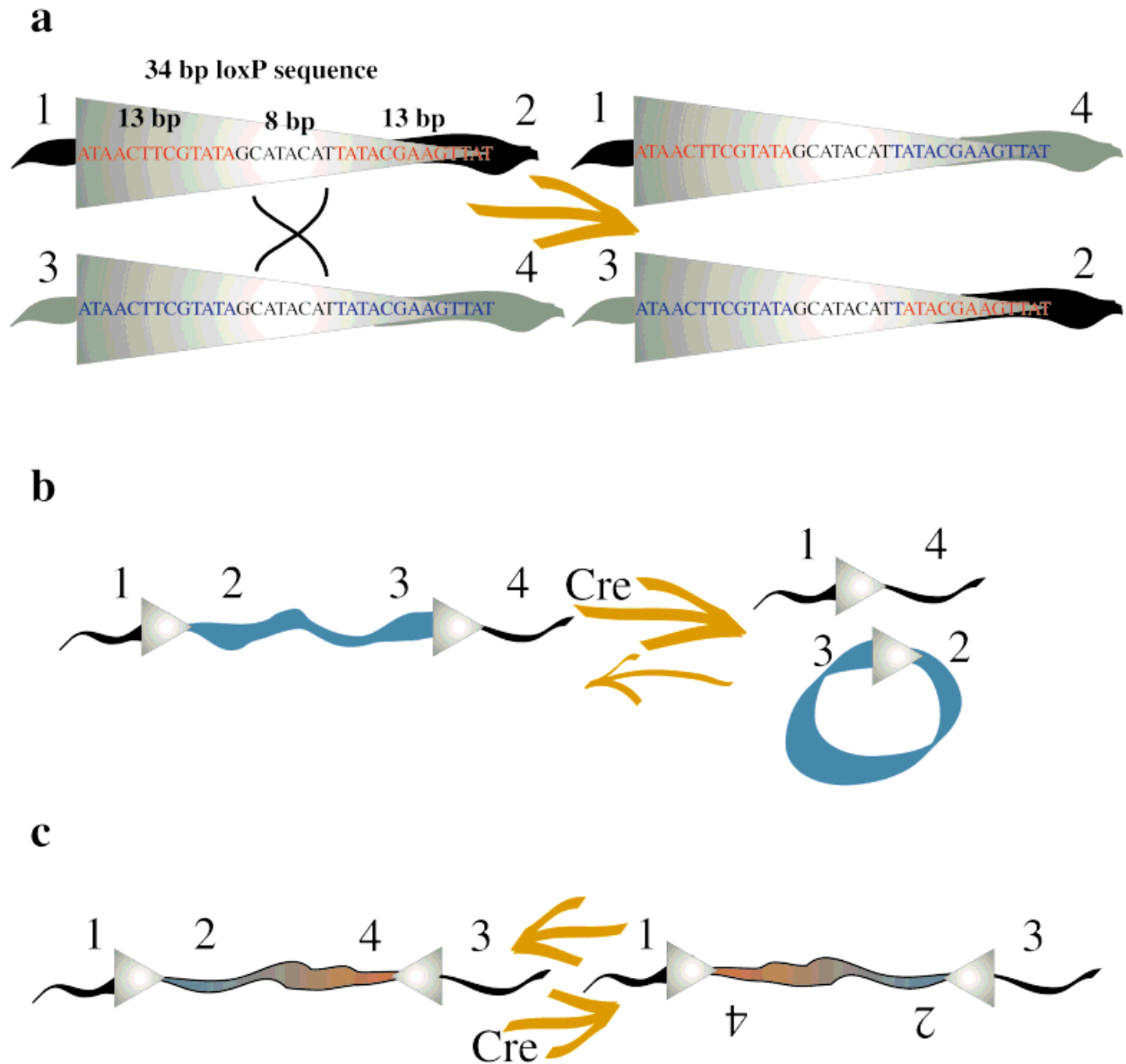
## BASIC PROPERTIES OF CRE RECOMBINASE

The Cre recombinase of the P1 bacteriophage belongs to the integrase family of site-specific recombinases. It is a 38 kD protein that catalyzes the recombination between two of its recognition sites, called *loxP* (Hamilton and Abremski, 1984). This is a 34 bp consensus sequence, consisting of a core spacer sequence of 8 bp and two 13 bp palindromic flanking sequences (Fig. 1A). The asymmetric core sequence defines an orientation to the *loxP* site. One of the great advantages of the Cre/*loxP* recombination system is that there is no need for additional co-factors or sequence elements for efficient recombination regardless of the cellular environment. Concerning the molecular mechanism of recombination, a single recombinase molecule binds to each palindromic half of a *loxP* site, then the recombinase molecules form a tetramer, thus bringing two *loxP* sites together (Voziyanov *et al.*, 1999). The recombination occurs within the spacer area of the *loxP* sites. The postrecombination *loxP* sites are formed from the two complementary halves of the prerecombination sites (Fig. 1A).

The first indication that the phage enzyme would work in eukaryotic cells emerged from in vitro cell culture experiments in the late '80s and early '90s (Sauer and Henderson, 1988; 1989; 1990). These experiments were followed by the demonstration that the Cre recombinase worked in the mouse as well when expressed from a transgene (Lakso *et al.*, 1992; Orban *et al.*, 1992).

An important issue is that a random occurrence of a specific 34 bp sequence requires a  $10^{18}$  bp length of DNA. The entire mammalian genome is only  $3 \times 10^9$  bp. Therefore it is highly unlikely that an exact *loxP* site is represented outside of the phage genome. Consequently, introducing it through transgenesis into the eukaryotic genome restricts the Cre-mediated recombination to the exogenous *loxP* site. The result of the Cre

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**FIG. 1.** Cre/loxP system. (A) Close-up of Cre recombinase-mediated recombination between two 34 bp loxP sites. Schematics of excision vs. integration (B) and inversion (C).

recombinase-mediated recombination depends on the location and orientation of the *loxP* sites. They can be located cis or trans. In the former situation, their orientation can be the same or the opposite (Fig. 1B,C). In the case of trans localization, the DNA strands involved can be linear or circular. The outcome of the recombination between *loxP* sites depends on the actual scenario concerning these variables. It could be excision or inversion of an intervening sequence in the case of cis *loxP* sites (Fig. 1B,C) or insertion of one DNA into another or translocation between two molecules (chromosomes) in the case of trans *loxP* sites.

ES cell and transgenic technologies quickly recognized (Gu *et al.*, 1993) the potential in the new tool and

started the development of complex strategies to achieve far more sophisticated genetic alterations than had been possible before. Now we have an enormous set of building blocks from which to select the desired combinations to achieve particular goals (Nagy, 1996). As far as imaginable genetic alterations are concerned, there is not too much left in the "impossible" category.

Cre recombinase is certainly one of the key tools that made many of the newly developed genome alteration technologies possible. For in vitro recombination between *loxP* sites in cis, excision works efficiently without any active selection if the Cre is transiently expressed from a strong promoter (Nagy *et al.*, 1998). The range of excision is surprisingly large. We successfully

excised an almost 400 kb genomic region with 50% efficiency with no selection for the excision event. This was done using our pCX-NLS-Cre vector (Mar and Nagy, unpublished), in which a Cre recombinase equipped with a nuclear localization signal was expressed with the exceptionally strong CMV enhancer/chicken  $\beta$ -actin promoter combination (Niwa *et al.*, 1991). For larger cis distances or for a trans type of recombination, the frequency is smaller, therefore a selection system is required to identify such events *in vitro* (Ramirez-Solis *et al.*, 1995). The selection is designed in such a way that the recombination event is needed to activate the expression of a positive selectable marker. The most frequently applied system is based on the bipartite Hprt mini-gene. The two halves of the gene are placed in the genome in such a way that the Cre-mediated recombination is the only possibility to bring the two pieces together and restore the function of the Hprt gene. The only limitation of this strategy is the requirement for an Hprt-deficient cell line as the subject of genetic manipulation. An alternative solution for a bipartite selectable marker is to split the expression construct between the promoter and the coding region. We preferred this strategy in the deletion study mentioned above and proved the principle. The third common selection system is based on the loss of a negative selectable marker (i.e., herpes virus thymidine kinase; R. Conlon, personal communication).

The *in vivo* Cre-mediated excision is also efficient if the recombinase is expressed at a sufficiently high level from a transgene. For most of the strategies applying Cre recombinase-mediated genome modifications, there is a requirement for reliable, high-fidelity, and specific transgenic expression of the Cre recombinase. The enormous effort of establishing Cre transgenic lines is gradually reaching the stage of at least partial satisfaction. This present stage certainly justifies the past, present, and future efforts to generate a universally useful panel of transgenic lines that completely cover the development and anatomy of the mouse like a jigsaw puzzle. Interestingly, during the attempts to fulfill the criteria for expression of the Cre recombinase, we even learned more about the "classical" transgenesis in the last few years. We realized that even a simple-sounding goal such as ubiquitous expression from a transgene is not easy to achieve (Hadjantonakis *et al.*, 1998; Lobe *et al.*, 1999). For tissue-specific expression, most cloned promoters are not complete and they are frequently subject to integration site variegation. Therefore their expression is frequently not reliable, mosaic in the target tissue or cell type, and they frequently pick up unexpected, ectopic expression. In spite of the above difficulties, the number of satisfying Cre recombinase transgenic lines is growing, as this issue of *Genesis* also illustrates.

To take advantage of the precise recombination mediated by the Cre enzyme, target *loxP* sites have to be introduced into the genome of the cells. There is not too much variation to this aspect; it can be achieved either by a simple random integration type of transgenesis or by targeted mutagenesis. For Cre action at least two *loxP*

sites are needed. Too many *loxP* sites, however, can lead to a problem of chromosomal instability. It was recently indicated that multiple copy integration of *loxP* sites and the presence of recombinase can lead to chromosome loss (Lewandoski and Martin, 1997). To stay on the safe side, it is important therefore to ensure that the minimum number of *loxP* sites is introduced into the cell for a certain strategy.

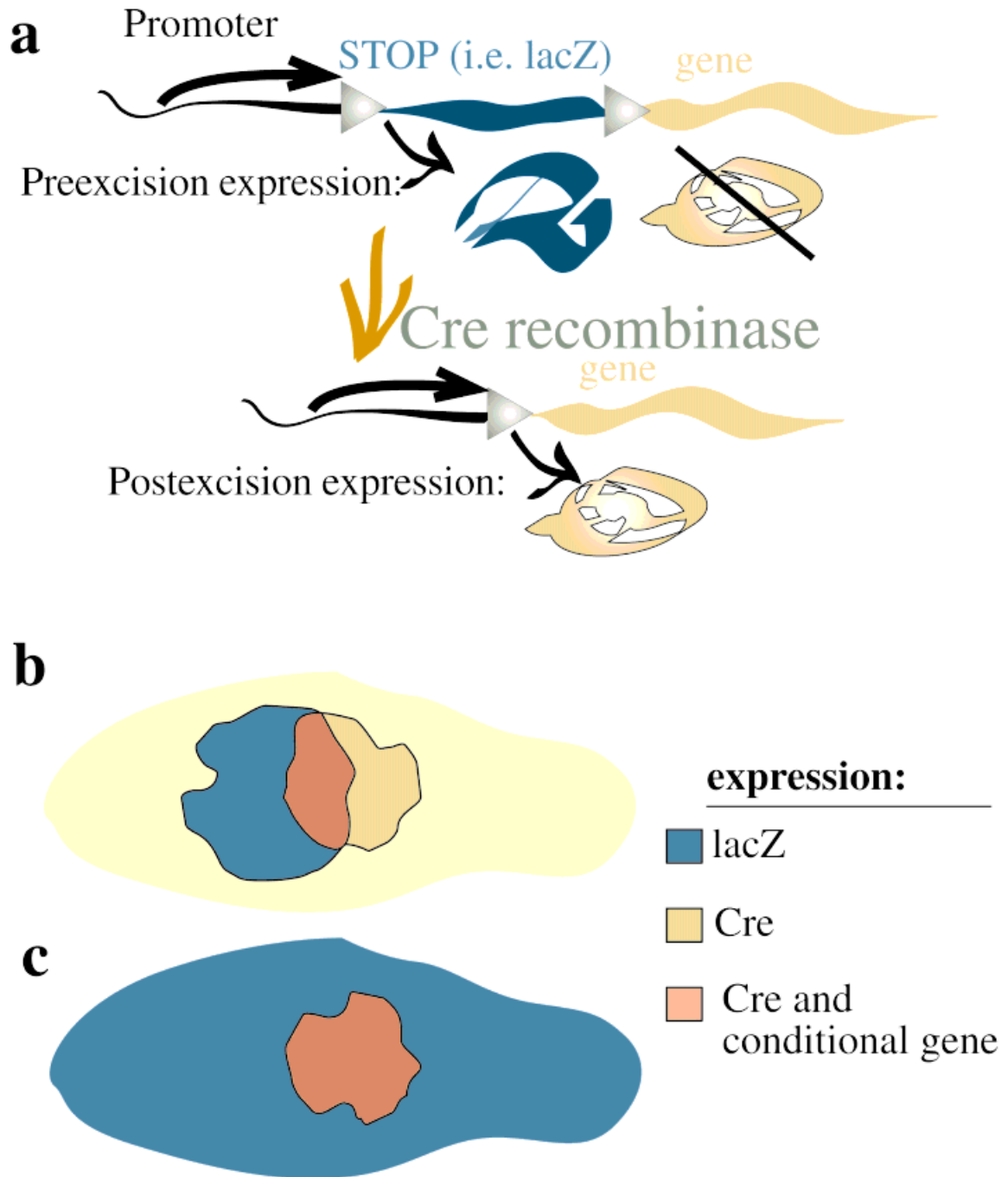
The means of introduction of the Cre recombinase into the cells bears a much higher number of variations. The usual *in vitro* introduction for transient expression or stable integration is electroporation or calcium precipitation. Electroporation in fact is surprisingly efficient. It can reach as high as 70% as measured by the Cre-mediated excision rate with the pCX-NLS-Cre vector (Nagy Lab, unpublished data). The favored method of introduction of Cre *in vivo* very much depends on the application. The simplest solution to achieve overall excision in the developing embryo is a cross between a *loxP* "provider" and a general Cre expresser transgenic line (Sakai and Miyazaki, 1997; Lallemand *et al.*, 1998). Another variation of this approach is pronuclear injection of a Cre expression vector, and the resultant transient Cre expression does its job during preimplantation development (Araki *et al.*, 1995). Injecting Cre RNA or protein itself may also work. These latter three means are obviously more tedious than simple mating, but the reward is that they do not introduce a Cre transgene into the system investigated. The main methodology for lineage/cell type-specific excision is the use of established transgenic lines expressing Cre under the control of a promoter with the required specificity. In certain applications, however, sporadic expression of Cre is the goal. Here retro- or adenovirus delivery systems have been developed. With these it is much harder to control the tissue specificity of Cre expression. As a solution, the combination of the avian retrovirus, TVA delivery system, and the recombinase systems provides the possibility of viral delivery of the Cre recombinase in a tissue- or cell-specific manner (Holland *et al.*, 1998). For the cellular entry of the virus, there is a requirement for a special avian receptor (tv-a), which has to be expressed in the mouse from a cell type-specific transgene to make those cells susceptible to virus infection.

All these different ways of introducing the Cre enzyme into the intracellular environment have their own pros and cons. Depending on the actual strategy and the questions asked, one of them will have the best fit.

## APPLICATIONS OF CRE RECOMBINASE IN MOUSE GENETICS

### In Traditional Transgenesis, Conditional Transgenes

Soon after the demonstration of Cre recombinase-mediated excision in tissue culture cells and *in vivo* in transgenic animals, the idea of Cre excision-conditional transgenesis surfaced (Lakso *et al.*, 1992). The basic concept evolved from the need to establish transgenic lines where the transgene product presented a survival prob-



**FIG. 2.** Cre recombinase conditional transgenesis. **(A)** A simple strategy for conditional transgene activation. Transgene activation region if both Cre and the conditional transgene are under the control of lineage/cell type-specific promoters **(B)** or the conditional transgene is ubiquitously expressed while the Cre transgene is specific **(C)**.

lem for the embryo or in juvenile development. The structure of the conditional transgene is simple: The promoter and the coding region for the gene of interest is separated by a *loxP*-flanked STOP region, which does not allow any transcription initiated from the promoter to read through and include the gene. Obviously, when

this region is removed by Cre-mediated excision, the gene can be expressed (Fig. 2A). The first demonstration of this idea was a lens-specific activation of the large T antigen through Cre-mediated excision (Lakso *et al.*, 1992).

There are two possible variations for conditional transgenesis. In the first, the conditional transgene is ex-



pressed under the control of a lineage/cell type-specific promoter and the Cre recombinase is under the control of either a lineage/cell type-specific promoter (Fig. 2B) or a ubiquitous one. In the second strategy, the conditional transgene is driven by a ubiquitous promoter (Fig. 2C) while the Cre expression is lineage/cell type-specific. Both approaches have their pros and cons. When both the Cre recombinase and the conditional transgenes are expressed with specific expression patterns, the activation of the silent transgene occurs only in the common area of expression (Fig. 2B). This strategy can increase the specificity of expression but limits the activation range of the transgene of interest to a subset of its particular promoter. Since in the second strategy the conditional transgene is expressed in a ubiquitous manner, it does not create a limitation. The activation of the transgene will be determined solely by the expression pattern of the Cre recombinase transgene (Fig. 2C). Certainly, this approach is the favored one due to the expectation that a large number of Cre transgenic lines with a wide range of expression patterns will be available soon. The production of a good, ubiquitously expressed conditional transgenic line with a single-copy transgene is the first requirement. It is then crossed with a panel of Cre transgenic lines to obtain a variety of lineage/cell type-specific activation of the transgene.

### In Traditional Targeting, Selectable Marker Removal

Soon after the demonstration of the *in vivo* excision capabilities of the Cre recombinase, the system was incorporated into gene-targeting strategies (Gu *et al.*, 1993). The simplest and most obvious application is the removal of the selectable marker from the targeted allele (Meyers *et al.*, 1998; Nagy *et al.*, 1998). There are valid concerns that the presence of a selectable marker expression cassette can influence the expression of the neighboring genes (Fiering *et al.*, 1995). Such interference can create a phenotype that does not reflect the role of the targeted gene at all and can lead to misinterpretation. For removal of the selectable marker, a "classical" targeting vector has to be just slightly modified: *loxP* sites have to be placed around the selectable marker. Another advantage of the selectable marker removal is the possibility of reusing the target vector to create homozygous knock-out ES cells (Rossant and Nagy, 1995; Abuin and Bradley, 1996; Nagy and Rossant, 1996). This strategy requires three rounds of selection in ES cells. The first step is the targeting of the gene of interest and the identification of heterozygous targeted ES cell line(s). Second is the selectable marker removal by transient expression of the Cre recombinase, and the third step is the retargeting of the line with the same target vector to knock out the remaining wild-type allele and obtain homozygous targeted ES cell line(s).

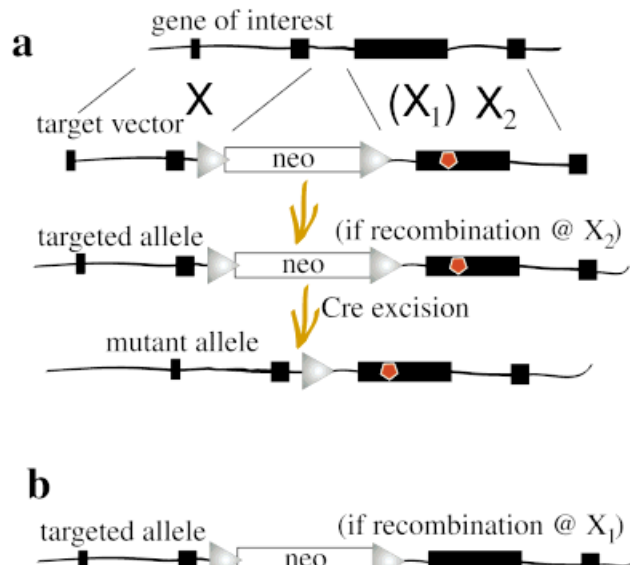
Inserting a *loxP* site-flanked selectable marker into an intronic area can provide a unique application that will be discussed in the conditional gene repair section.

### Subtle Change and Hypomorphic Alleles

The frequency of homologous recombination using a given target vector depends on many factors, including the amount of mismatch between the arms of homology and the targeted gene. Although the targeting frequency may negatively correlate with the number of mismatches, the targeting is still feasible with well-defined and localized changes in the homologous arms. This was one of the requirements for introducing subtle genetic changes into a gene of interest. The other requirement was the post-targeting removal of the selectable marker, which then allows the functioning of the targeted allele, provided nothing but the alteration embedded in the homology arm was introduced. Here again the *loxP*-flanked selectable marker placed into a noncoding region (Fig. 3A) provides the solution (Nagy *et al.*, 1998; Reichardt *et al.*, 1998; Wang *et al.*, 1999). Depending on the site of recombination, the homology arm-embedded alteration is either introduced or not introduced into the targeted allele. The latter is useful, since it serves as a control that the remaining *loxP* site does not create a disturbance in the gene function after the selectable marker is removed. Interestingly, this allele without the marker removal (Fig. 3B) can provide a useful tool for learning more about the function of the gene of interest. If the marker was designed properly, the allele containing the marker may be a "knock-down" allele, representing a lower than normal level of expression. These hypomorphic alleles have proven to be very informative in several cases exploring multiple functions of a gene (Meyers *et al.*, 1998; Nagy *et al.*, 1998). One of the neomycin resistance (*neo<sup>R</sup>*) gene constructs, pPNT (Tybulewicz *et al.*, 1991), which has frequently been used for identifying cell lines that integrated the target vector, has a cryptic splice acceptor site between the PGK promoter and the *neo<sup>R</sup>* coding region. This creates an abnormal splicing event when the PGK-promoter-*neo<sup>R</sup>* cassette is sitting in an intron with the same transcriptional orientation as the gene. Although the splicing around the insertion in the two examples (Meyers *et al.*, 1998; Nagy *et al.*, 1998) creates a normal transcript, the level of the mRNA is lowered to about 40% of the wild type. The combination of such a hypomorphic allele with either a null or wild-type allele gives a series of hypomorphic genotypes, adding up to 20%, 40%, 50%, and 70% of the level of two wild-type alleles (Nagy *et al.*, 1998). Inserting the *neo<sup>R</sup>* cassette in a reverse orientation to the gene creates a far more severe compromise in the level of mRNA. The *neo<sup>R</sup>* coding region read backwards has an almost completely efficient splice acceptor site (Jacks *et al.*, 1994). This creates an insertion into the original transcript. In addition, in the cases reported, it also significantly compromises the mRNA level (Jacks *et al.*, 1994; Carmeliet *et al.*, 1996).

### Cre Excision Conditional Gene Knock-Out

The most popular idea and perhaps one of the most powerful approaches developed from the combination

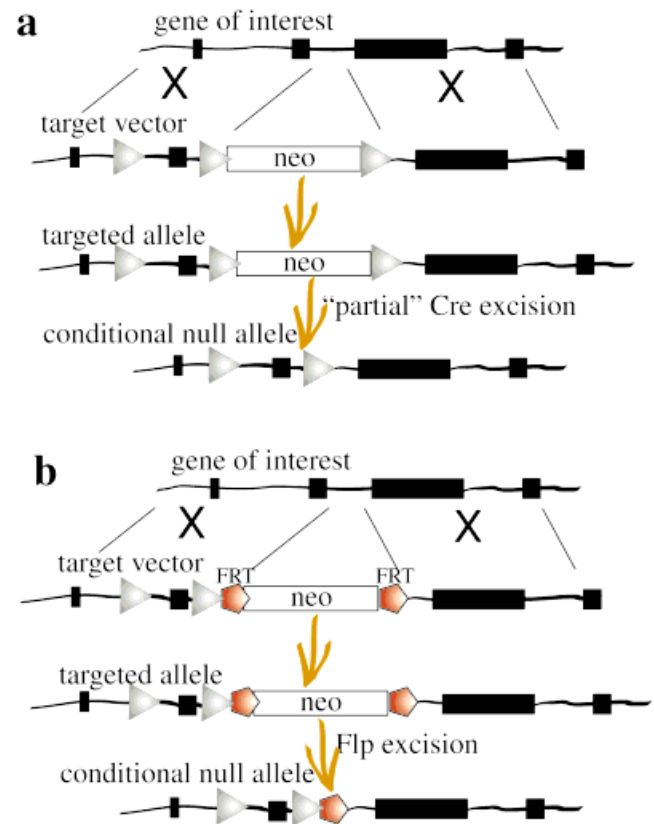


**FIG. 3.** The strategy of introducing a subtle change (A) and a hypomorphic allele (as a “by-product”) (B) into a gene.

of gene targeting and site-specific recombination is that of lineage/cell type-specific knock outs (Tsien *et al.*, 1996; Takeda *et al.*, 1998). The basic idea is very simple: use gene targeting to insert two *loxP* sites around a functionally essential part of the gene of interest, thus making a minimal change that leaves the gene completely functional. Embryos or animals homozygous for such an allele and also transgenic for a lineage/cell type-specific Cre recombinase transgene will have completely normal gene function outside of the Cre expression/excision cell lineage. In contrast, within the Cre excision lineage the gene will be knocked out.

This simple idea, however, is full of traps. One should be aware of these before going into a serious design and project. The first problem is the proper insertion of *loxP* sites around an essential functional part of the gene of interest. The selection of the conditional deletion is critical to achieve a null allele upon Cre excision. The safest strategy is to flank the entire gene with *loxP* sites, but in most of the cases it is technically impossible. The typical selection of a vital exon to be removed by Cre is the translation start, which always has a risk that after excision the allele will produce an altered product with a residual or altered function.

The other problem that requires serious consideration is the strategy for inserting the *loxP* sites into the gene and keeping the modified allele fully functional. The selectable marker has to be removed and two *loxP* sites have to be left behind. There are examples of successful use of three *loxP* sites in a target vector (Gu *et al.*, 1994). Two of the sites flank the selectable marker and the third one is placed into one of the homology arms in a way that this one and the selectable marker are around the functionally essential part of the gene. After successful



**FIG. 4.** Strategies for creating a Cre excision-conditional knock-out allele by using three *loxP* sites (A) and by combining the Cre/*loxP* with the Flp/FRT system (B).

targeting of the *loxP* sites, the Cre recombinase is transiently expressed in the ES cells, and the next task is to identify clone(s) in which a particular partial excision occurred, namely, when only the selectable marker is removed but the essential part remained (Fig. 4A). This is usually not easy to achieve and the success might be gene dependent. The alternative strategy is the use of the Flp recombinase system to remove the selectable markers (Meyers *et al.*, 1998) (Fig. 4B). The advantage of this strategy over the previous one is that it can be done in vivo, after germline transmission of the targeted allele, and the excision does not disturb the *loxP*-flanked region. The disadvantage is that the Flp system is far less efficient than the Cre. It means that identifying successfully excised alleles requires screening of a relatively large number of animals that are double transgenic for Flp (Dymecki and Tomasiewicz, 1998) and the targeted allele. The newly developed enhanced Flp recombinase, Flpe (Buchholz *et al.*, 1998), however, holds promise that the efficiency of this site-specific recombinase system will get closer to that of the Cre/*loxP*.

The current low number of lineage/cell type-specific Cre transgenic lines also represents limitations in the application of this technology. It is likely, however, that in the near future there will be enough lines that will

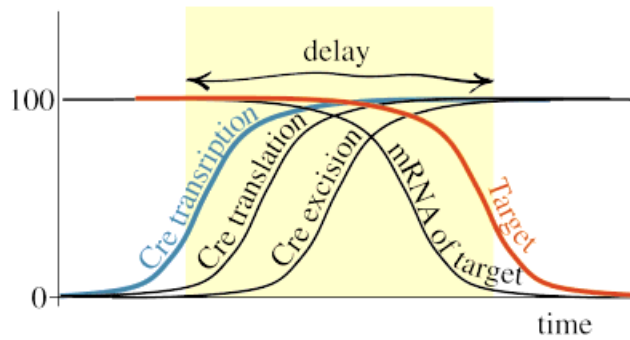


FIG. 5. Multilevel delay of the Cre recombinase effect.

cover the important developmental processes and functional/anatomical structures. There is a need therefore to identify a large number of gene promoters that are characteristic for cell types, lineages, and developmental events. The “large number” will likely be important here, since not all will be appropriate for driving Cre recombinase at a sufficient level and required specificity. There will be many lines in which the Cre-mediated excision will not be 100% efficient. If such lines are used for lineage/cell type-specific knock-outs, the partial excision can create extreme difficulties in analyzing the phenotypes. Partial excision creates mosaicism with a variable size of the wild-type compartment, and in most cases this could be translated into a variation of the observed phenotype, making the interpretation difficult or impossible.

On the other hand, it is easy to envision phenotype analyses or types of questions where the mosaicism of mutant versus wild-type cells, within a lineage- or cell type-restriction, could serve as an informative tool (Betz *et al.*, 1996). An obvious need for such an approach is the ability to distinguish the two cell populations at a cellular level, but with a clever design it is feasible to get reporter activation joined to simultaneous inactivation of a gene. Therefore the Cre transgenic lines that are not 100% efficient in the target lineage could be useful for mosaic studies. There are ever-stronger indications for a renaissance of chimera and mosaic studies in analyzing phenotypes of targeted mutations (Lobe and Nagy, 1998; Rossant and Spence, 1998). To be able to do this in a lineage-restricted manner would be just as important an extension of the chimera/mosaic studies as the conditional knock-outs are on the “classical” knock-outs. This is important to keep in mind before these “lousy” Cre lines become the victims of the first burst of disappointment.

Regardless of what job the recombinase has to do on the mouse gene of interest, there are two important things to consider. First, the Cre recombinase-mediated excision is a stochastic event. It does not happen at the same time in all the cells of the target lineage. The number of cells that undergo Cre recombination follows a sigmoid curve in the function of time, which can

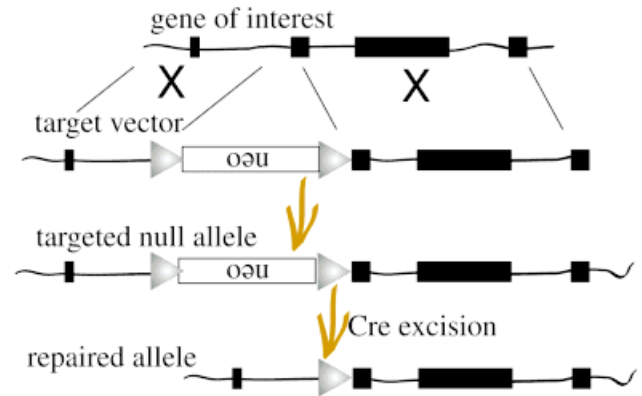


FIG. 6. Strategy for Cre excision-conditional gene repair.

saturate at 100% or at lower levels. The parameters of the sigmoid curve depend on the expression characteristics of the Cre enzyme and the availability of the *loxP*-flanked segment. The most important thing to remember is that the Cre-mediated excision takes time. However, this is not the only process that creates a gap between the onset of Cre expression and the effect of Cre-mediated excision. The second important thing is the following: If Cre is expressed under the control of a lineage-specific promoter, first the transcription initiates, which leads to the accumulation of the messenger RNA. Then the translated protein has to be built up to the steady state level. These accumulations follow more or less sigmoid-type curves with gaps separating them, as shown in Fig. 5 as a hypothetical representation. As the concentration of the Cre protein builds up, the probability of recombination between the target *loxP* sites increases. The resultant excision changes the target gene. In the case of loss-of-function, it gradually eliminates the template for the mRNA production so the mRNA produced from the gene of interest decreases (Fig. 5). And now, added to the parameters that build up to the Cre excision event, the same parameters are added in reverse for the target gene, namely the mRNA and protein half-lives. The total delay between the onset of Cre transcription and the actual biological effect is determined by multiple factors and could vary between a few hours and days. It might be too long to study quickly changing, dynamic events.

### Cre Excision Conditional Gene Repair

As mentioned earlier, the insertion of the *neo<sup>R</sup>* selectable marker into an intron causes a significant disturbance in gene functions. This is especially true when the *neo<sup>R</sup>* gene is in reverse orientation relative to the targeted gene (Fig. 6). If nothing else is modified in the targeted allele and the *neo<sup>R</sup>* selectable marker is flanked by *loxP* sites, then this highly compromised targeted allele can be repaired by a Cre-mediated excision (Nagy *et al.*, 1998). This repair developed into a concept of Cre-mediated gene repair, which can be done even in a lineage/cell type-specific fashion. The concept is to



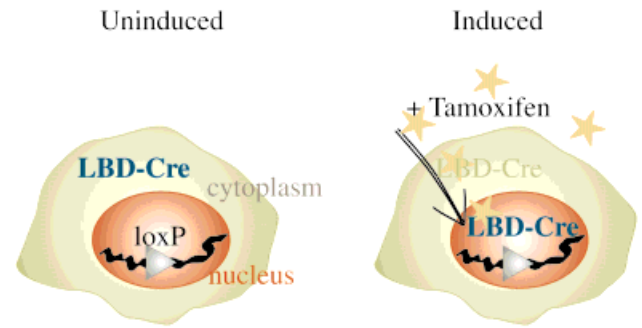
knock out a gene of interest in a way that is repairable, then analyze the phenotype of the homozygous embryos/animals for this allele and identify the primary lineage affected. After identifying the phenotype, the mice are crossed to a Cre recombinase line, with specific Cre transgene expression in the lineage identified as primarily affected, to obtain double transgenic embryo/animals having the repairable allele in a homozygous form. The Cre recombinase will repair the targeted allele in the lineage of specificity and rescue the primary phenotype allowing the embryo/animal to display the chronologically second one. Such an approach has the power to explore multiple functions of a gene, which is a fairly common phenomenon. Since the task here is the rescue of a lineage/cell type-specific phenotype, it might not require a 100% efficient repair in the lineage. Chimera experiments show that most of the lineages can function normally even with a fairly significant level of mutant cell contribution. Therefore the expectation is that the lineage-specific gene repair approach will not be very sensitive to the mosaic type of excision achieved by a non-ideal Cre transgene.

To achieve a repairable gene knock-out is not an obvious task for most genes. The intronic insertion of a strong, interfering splice acceptor might not work for all situations. There is a need to develop other strategies, such as putting in a removable 5' untranslated region, that might provide alternatives.

It is not surprising that the questions addressed with lineage/cell type-specific gene repair are different than with knock-outs. Rather than providing alternative approaches, these two concepts complement each other in dissecting complex phenotypes.

#### Combination With an Inducible System: Tamoxifen

Even in the ideal future, when all the endogenous gene regulatory elements are characterized and are available for use in transgenesis, driving Cre with those will retain limitations. One of them is the onset of expression, which would be desirable to control independently from the endogenous regulatory elements. Therefore several laboratories are intensively working on combining the Cre recombinase system with inducible gene expression systems. To date the two most promising inducible systems are the tamoxifen and the RU-486 (Brocard *et al.*, 1998; Kellendonk *et al.*, 1999). Each takes advantage of the nuclear localization capability of the estrogen or progesterone receptor ligand-binding domains in the presence of a ligand. The Cre recombinase is fused to a mutant ligand-binding domain, which lost its ability to bind endogenous estrogen or progesterone but still binds tamoxifen (an estrogen antagonist) or RU486 (a synthetic steroid), respectively. In the presence of synthetic ligand, the Cre fusion protein translocates into the nucleus where it executes its function (Fig. 7). The published data show that it is possible to obtain inducibility of the Cre-mediated excision, but in most cases it was only partial (Danielian *et al.*, 1998). The system



**FIG. 7.** Mechanism of action of steroid ligand-binding domain (LBD): Cre fusion proteins.

requires further tuning to achieve better inducibility for broad application. The obvious and ultimate goal is to combine inducibility with lineage/cell type-specific expression of the Cre recombinase, where the “switch on” could be independently controlled from the lineage specificity.

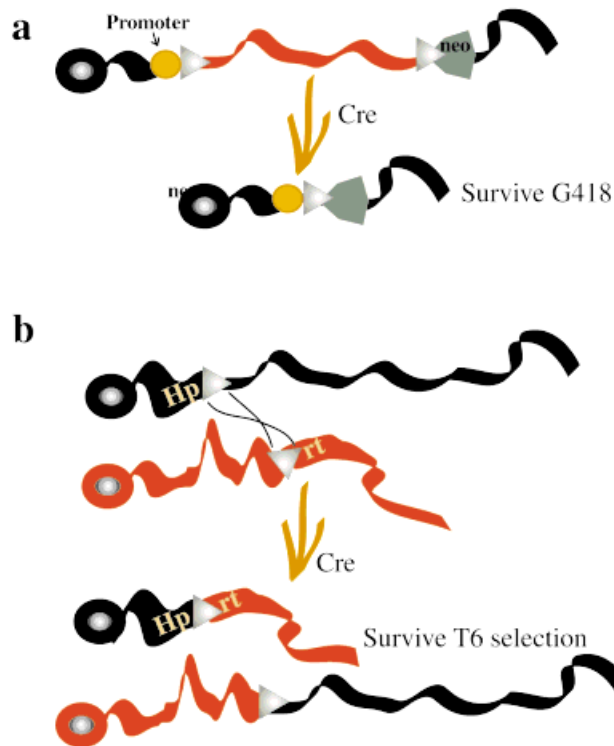
#### Defined Chromosomal Aberrations: Deletion, Inversion, Translocation

There is an important area of Cre recombinase applications in manipulating the mouse genome, which at the moment has not too much to do with transgenic lines expressing the enzyme. However, it is important enough to spend a few sentences here. This is the area of creating large chromosomal aberrations: deletions (Fig. 8A), translocations (Fig. 8B), and inversions (Ramirez-Solis *et al.*, 1995; Zheng *et al.*, 1999). The Cre recombinase works between large distances within a chromosome or in trans between different chromosomes (Smith *et al.*, 1995). As expected, the recombination frequency is significantly lower in these situations, but it is still possible to identify these events *in vitro* in ES cells, where millions of cells are available for selection. Because of the low frequency event, the selection has to be very tight, which allows survival of cells only if the desired Cre-mediated event occurred. One strategy uses an Hprt-negative cell line where the designed chromosomal change restores Hprt expression of the cell, which then survives the HAT selection. This was used to create large deletions and inversions (Ramirez-Solis *et al.*, 1995; Zheng *et al.*, 1999). The other design utilizes the herpes virus thymidine kinase (tk) negative selectable marker, where the loss of the marker gene renders the cell to survive in FIAU selection. This method was applied to achieve large defined chromosomal deletions (R. Conlon, personal communication).

#### Pop-In Combinations With Transgenesis, Targeting, Gene Trap

The utilization of trans Cre-mediated recombination led to designs that allow site-specific insertion of a transgene into the genome. The basic mechanism here is recombination between a circular DNA (for example, a plasmid)

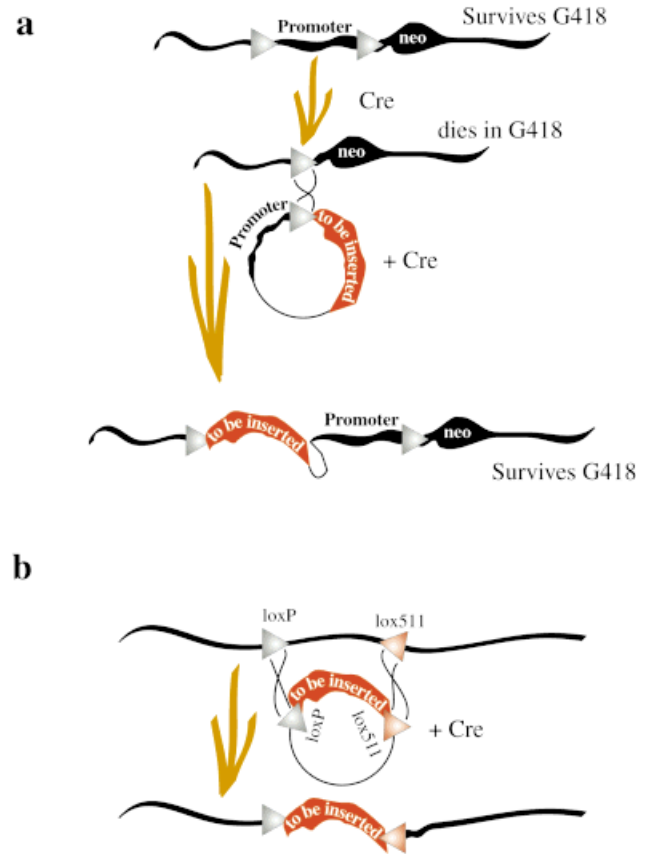




**FIG. 8.** Cre recombinase-mediated site-specific chromosomal alterations. (A) Large chromosomal deletion by targeting *loxP* sites to the two ends of the region of interest. (B) Reciprocal translocation by interchromosomal recombination.

and a linear DNA (a chromosome). One possible design to achieve this is depicted in Fig. 9A. This figure also shows the obstacle in this approach. If simple *loxP* sites are used, then the intermolecular recombination creates two *loxP* sites in the chromosome, which will be placed in close vicinity and provide a *cis* substrate for intramolecular recombination (excision). The latter occurs at much higher efficiency than the former, so the equilibrium favors the excised stage over the pop-in stage. The ability to screen for events using a large number of ES cells helps out this situation too. If a *loxP* site was previously placed into the genome and a *loxP*-containing circular plasmid is co-electroporated with a Cre recombinase expression vector into a million ES cells (one electroporation), then, after the transient expression of the enzyme is diminished, dozens of cells will still be in the disfavored "pop-in" stage. Now it is a matter of a strict selection system to identify those cells and derive clones from them. One design to achieve this is to have a promoterless positive selectable marker preceded by a *loxP* site, which is placed into the genome. Cre-mediated insertion of the circular plasmid delivers a promoter before the selectable marker and drives its expression. As long as the promoter remains there, the cell survives the selection (Fig. 9A).

A second strategy for achieving site-specific insertion of a DNA segment is to modify the biased equilibrium



**FIG. 9.** Strategies for Cre recombinase-mediated site-specific DNA insertion. Insertion using two single *loxP* sites (A) and using the "double lox recombination" (B) (Soukharev *et al.*, 1999).

between the pop-in versus pop-out states. The Cre recombinase has a tolerance for recognizing mutant *loxP* sites if the mutation affects only one nucleotide at the marginal part of the site. The recombination occurs with close to normal frequency if both *loxP* sites are mutant for one nucleotide. If more than one nucleotide is mutated, the recombination between such sites and a normal or any mutant site is far less efficient. There are now well-documented examples that take advantage of these characteristics of Cre-mediated recombination (Araki *et al.*, 1997; 1999). This approach does not require as strict a selection as in the previous strategy, but after the insertion is achieved the segment cannot be removed easily.

The third strategy is based on the observation that there is an alternative recognition site, *lox511* for the Cre recombinase, which differs from the normal *loxP* site in the spacer sequence. Cre efficiently recombines between two *lox511* sites but at a low efficiency between a *loxP* and a *lox511* site. Here a *loxP* and a *lox511* site are placed into the genome in close vicinity. Transient Cre expression in the cells catalyzes recombination between a vector that has *loxP* and *lox511* sites flanking the segment that is to be inserted (Soukharev *et al.*,

1999). Recombination occurs only between the pair of *loxP* and the pair of *lox511* sites but not between the two, as shown in Fig. 9B. After insertion, the pop-out would require a recombination between a *loxP* and a *lox511* site, which occurs with a low efficiency. Thus the pop-in state is favored. The attractive feature of this strategy is that the popped-in *loxP/lox511*-flanked segment of DNA stays in position, even when Cre recombinase is still around. Therefore it is possible to integrate the Cre recombinase itself into a defined site of the genome, which would have a very important role in expanding the collection of lineage/cell type-specific Cre expressor transgenic lines by combining the Cre recombinase pop-in with gene-trap strategies.

## MAKING THE CRE TRANSGENIC LINES

The power of the Cre recombinase-based strategies will very much depend on the collection of Cre transgenic lines expressing the recombinase with a high enough level and specificity to achieve the desired type of genome alterations. In spite of the very promising fact that we now have quite a few very satisfying results, the huge effort of producing and characterizing such transgenic lines showed that production of the final collection will not be an easy task. It is obvious that we cannot rely only on classical transgenesis utilizing the limited set of identified and, most of the time, only partially characterized promoter elements. There is lots of room for new ideas. For example, the gene targeting-based Cre "knock-in" allows us to drive Cre by endogenous gene regulation. There are ways to combine gene-trap strategies with pop-ins and utilize the expression of trapped endogenous regulations for expressing the Cre recombinase. The only disadvantage of both gene-targeting and gene-trap-based Cre knock-ins is that they create a mutation of an endogenous gene, which may interfere with further applications. However, the advantages of making all the gene regulations accessible for Cre expression and the likeliness of more reliable expression than a randomly integrating transgene leaves no question about the impact of using these means to produce transgenic lines expressing Cre in a lineage/cell type-specific manner.

## TESTING THE CRE TRANSGENIC LINES

An important issue is testing the transgenic lines for the efficiency and specificity of Cre-mediated recombination in a standard and most simple way possible at the individual cell level. The idea here is to activate a reporter gene expression if Cre-mediated recombination occurs. For this a single copy transgene should be produced, which has a ubiquitously active promoter, followed by a *loxP*-flanked STOP region that does not allow the expression of a reporter gene placed after. Upon Cre excision, the reporter comes under the direct control of the promoter and starts to be expressed. Interestingly, such a simple task as ubiquitous expression of a transgene was not easy to achieve. One solution was targeting the

Rosa-26 gene-trap integration site with a Cre excision conditional *lacZ* reporter (Mao *et al.*, 1999; Soriano, 1999). The other solution was an extensive selection in ES cells for permissive single-copy integration sites of a CMV enhancer/chicken  $\beta$ -actin promoter-driven construct, followed by ES-mediated transgenesis. The latter (Z/AP) (Lobe *et al.*, 1999) utilized two reporters. The *lacZ* reporter marks cells before excision occurs, and the heat-resistant human placental alkaline phosphatase (hAP) marks post-excision cells.

The Cre-mediated recombination frequency might vary between genomic target locations. Testing the Cre transgenic lines for recombination efficiency and specificity with more than one reporter line could inform of such variability.

## A PERSONAL MESSAGE

A panel of working Cre recombinase transgenic lines with a spectrum of expression specificity is considered a universal reagent that can be used in many different projects. Because of this nature, it should be created as a joint effort of the scientific community interested in applying the technology. Initiated by this concept, a couple of years ago I started organizing a database for the use of everyone interested and with the very valuable help of those who agreed with this concept. After the first years of being mostly a skeleton of plans, there is now some real meat in the database (<http://www.mshri.on.ca/nagy/Cre.html>). We are all certain that with a joint effort the collection of Cre transgenic lines will develop into a strong and efficient machine to dissect phenotypes of mutations introduced into the mouse genome and to create better models for human disease. These can ultimately lead to a better understanding of human disease and eventually to cures.

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## LITERATURE CITED

- Abuin A, Bradley A. 1996. Recycling selectable markers in mouse embryonic stem cells. *Mol Cell Biol* 16:1851-1856.
- Araki K, Araki M, Miyazaki J, Vassalli P. 1995. Site-specific recombination of a transgene in fertilized eggs by transient expression of Cre recombinase. *Proc Natl Acad Sci USA* 92:160-164.
- Araki K, Araki M, Yamamura K. 1997. Targeted integration of DNA using mutant lox sites in embryonic stem cells. *Nucleic Acids Res* 25:868-872.
- Araki K, Imaizumi T, Sekimoto T, Yoshinobu K, Yoshimuta J, Akizuki M, Miura K, Araki M, Yamamura K. 1999. Exchangeable gene trap using the Cre/mutated lox system [In Process Citation]. *Cell Mol Biol (Noisy-Le-Grand)* 45:737-750.

- Betz UA, Vosshenrich CA, Rajewsky K, Muller W. 1996. Bypass of lethality with mosaic mice generated by Cre-*loxP*-mediated recombination. *Curr Biol* 6:1307-1316.
- Brocard J, Feil R, Chambon P, Metzger D. 1998. A chimeric Cre recombinase inducible by synthetic, but not by natural ligands of the glucocorticoid receptor. *Nucleic Acids Res* 26:4086-4090.
- Buchholz F, Angrand PO, Stewart AF. 1998. Improved properties of FLP recombinase evolved by cycling mutagenesis [see comments]. *Nat Biotechnol* 16:657-662.
- Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, Fahrig M, Vandenhoec A, Harpal K, Eberhardt C, Declercq C, Pawling J, Moons L, Collen D, Risau W, Nagy A. 1996. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* 380:435-439.
- Danielian PS, Muccino D, Rowitch DH, Michael SK, McMahon AP. 1998. Modification of gene activity in mouse embryos in utero by a tamoxifen-inducible form of Cre recombinase. *Curr Biol* 8:1323-1326.
- Dymecki SM, Tomasiewicz H. 1998. Using Flp-recombinase to characterize expansion of Wnt1-expressing neural progenitors in the mouse. *Dev Biol* 201:57-65.
- Fiering S, Epner E, Robinson K, Zhuang Y, Telling A, Hu M, Martin DI, Enver T, Ley TJ, Groudine M. 1995. Targeted deletion of 5'HS2 of the murine beta-globin LCR reveals that it is not essential for proper regulation of the beta-globin locus. *Genes Dev* 9:2203-2213.
- Gu H, Marth JD, Orban PC, Mossman H, Rajewsky K. 1994. Deletion of a DNA polymerase beta gene segment in T cells using cell type-specific gene targeting [see comments]. *Science* 265:103-106.
- Gu H, Zou YR, Rajewsky K. 1993. Independent control of immunoglobulin switch recombination at individual switch regions evidenced through Cre-*loxP*-mediated gene targeting. *Cell* 73:1155-1164.
- Hadjantonakis AK, Gertsenstein M, Ikawa M, Okabe M, Nagy A. 1998. Generating green fluorescent mice by germline transmission of green fluorescent ES cells. *Mech Dev* 76:79-90.
- Hamilton DL, Abremski K. 1984. Site-specific recombination by the bacteriophage P1 *lox*-Cre system. Cre-mediated synapsis of two *lox* sites. *J Mol Biol* 178:481-486.
- Holland EC, Hively WP, DePinho RA, Varmus HE. 1998. A constitutively active epidermal growth factor receptor cooperates with disruption of G1 cell-cycle arrest pathways to induce glioma-like lesions in mice. *Genes Dev* 12:3675-3685.
- Jacks T, Shih TS, Schmitt EM, Bronson RT, Bernards A, Weinberg RA. 1994. Tumour predisposition in mice heterozygous for a targeted mutation in Nf1. *Nat Genet* 7:353-361.
- Kellendonk C, Troche F, Casanova E, Anlag K, Opherk C, Schutz G. 1999. Inducible site-specific recombination in the brain. *J Mol Biol* 285:175-182.
- Lakso M, Sauer B, Mosinger B Jr., Lee EJ, Manning RW, Yu SH, Mulder KL, Westphal H. 1992. Targeted oncogene activation by site-specific recombination in transgenic mice. *Proc Natl Acad Sci USA* 89:6232-6236.
- Lallemand Y, Luria V, Haffner-Krausz R, Lonai P. 1998. Maternally expressed PGK-Cre transgene as a tool for early and uniform activation of the Cre site-specific recombinase. *Transgenic Res* 7:105-112.
- Lewandoski M, Martin GR. 1997. Cre-mediated chromosome loss in mice. *Nat Genet* 17:223-225.
- Lobe CG, Koop KE, Kreppner W, Lomeli H, Gertsenstein M, Nagy A. 1999. Z/AP, a double reporter for Cre-mediated recombination. *Dev Biol* 208:281-292.
- Lobe CG, Nagy A. 1998. Conditional genome alteration in mice. *Bioessays* 20:200-208.
- Mao X, Fujiwara Y, Orkin SH. 1999. Improved reporter strain for monitoring Cre recombinase-mediated DNA excisions in mice. *Proc Natl Acad Sci USA* 96:5037-5042.
- Meyers EN, Lewandoski M, Martin GR. 1998. An Fgf8 mutant allelic series generated by Cre- and Flp-mediated recombination. *Nat Genet* 18:136-141.
- Nagy A. 1996. Engineering the mouse genome. In: Lonai P, editor. *Mammalian development*. Amsterdam: Harwood Academic Publishers. p 339-371.
- Nagy A, Moens CB, Ivanyi E, Pawling J, Gertsenstein M, Hadjantonakis A-K, Purity M, Rossant J. 1998. Dissecting the role of *N-myc* in development using a single targeting vector to generate a series of alleles. *Curr Biol* 8:In press.
- Nagy A, Rossant J. 1996. Targeted mutagenesis: analysis of phenotype without germ line transmission. *J Clin Invest* 97:1360-1365.
- Niwa H, Yamamura K, Miyazaki J. 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* 108:193-199.
- Orban PC, Chui D, Marth JD. 1992. Tissue- and site-specific DNA recombination in transgenic mice. *Proc Natl Acad Sci USA* 89:6861-6865.
- Ramirez-Solis R, Liu P, Bradley A. 1995. Chromosome engineering in mice. *Nature* 378:720-724.
- Reichardt HM, Kaestner KH, Wessely O, Gass P, Schmid W, Schutz G. 1998. Analysis of glucocorticoid signalling by gene targeting. *J Steroid Biochem Mol Biol* 65:111-115.
- Rossant J, McMahon A. 1999. "Cre"-ating mouse mutants—a meeting review on conditional mouse genetics. *Genes Dev* 13:142-145.
- Rossant J, Nagy A. 1995. Genome engineering: the new mouse genetics. *Nat Med* 1:592-594.
- Rossant J, Spence A. 1998. Chimeras and mosaics in mouse mutant analysis. *Trends Genet* 14:358-363.
- Sakai K, Miyazaki J. 1997. A transgenic mouse line that retains Cre recombinase activity in mature oocytes irrespective of the Cre transgene transmission. *Biochem Biophys Res Commun* 237:318-324.
- Sauer B, Henderson N. 1988. Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1. *Proc Natl Acad Sci USA* 85:5166-5170.
- Sauer B, Henderson N. 1989. Cre-stimulated recombination at *loxP*-containing DNA sequences placed into the mammalian genome. *Nucleic Acids Res* 17:147-161.
- Sauer B, Henderson N. 1990. Targeted insertion of exogenous DNA into the eukaryotic genome by the Cre recombinase. *New Biol* 2:441-449.
- Smith AJ, De Sousa MA, Kwabi-Addo B, Heppell-Parton A, Impey H, Rabbitts P. 1995. A site-directed chromosomal translocation induced in embryonic stem cells by Cre-*loxP* recombination [published erratum appears in *Nat Genet* 1996 Jan;12(1):110]. *Nat Genet* 9:376-385.
- Soriano P. 1999. Generalized lacZ expression with the ROSA26 Cre reporter strain [letter]. *Nat Genet* 21:70-71.
- Soukharev S, Miller JL, Sauer B. 1999. Segmental genomic replacement in embryonic stem cells by double *lox* targeting. *Nucleic Acids Res* 27:e21.
- Takeda K, Kaisho T, Yoshida N, Takeda J, Kishimoto T, Akira S. 1998. Stat3 activation is responsible for IL-6-dependent T cell proliferation through preventing apoptosis: generation and characterization of T cell-specific Stat3-deficient mice. *J Immunol* 161:4652-4660.
- Tsien JZ, Chen DF, Gerber D, Tom C, Mercer EH, Anderson DJ, Mayford M, Kandel ER, Tonegawa S. 1996. Subregion- and cell type-restricted gene knockout in mouse brain. *Cell* 87:1317-1326.
- Tybulewicz VL, Crawford CE, Jackson PK, Bronson RT, Mulligan RC. 1991. Neonatal lethality and lymphopenia in mice with a homozygous disruption of the *c-abl* proto-oncogene. *Cell* 65:1153-1163.
- Vozizyanov Y, Pathania S, Jayaram M. 1999. A general model for site-specific recombination by the integrase family recombinases. *Nucleic Acids Res* 27:930-941.
- Wang Y, Spatz MK, Kannan K, Hayk H, Avivi A, Gorivodsky M, Pines M, Yayon A, Lonai P, Givol D. 1999. A mouse model for achondroplasia produced by targeting fibroblast growth factor receptor 3. *Proc Natl Acad Sci USA* 96:4455-4460.
- Zheng B, Sage M, Cai WW, Thompson DM, Tavsanli BC, Cheah YC, Bradley A. 1999. Engineering a mouse balancer chromosome. *Nat Genet* 22:375-378.